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Cross-Talk in the Female Rat Mammary Gland: Influence of Aryl Hydrocarbon Receptor on Estrogen Receptor Signaling

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Running title: Aryl Hydrocarbon- and Estrogen receptor cross-talk

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Abstract

Background: Cross-talk between Aryl hydrocarbon receptor (AHR) and Estrogen receptor (ER) plays a major role in female reproductive organs.

Objectives: This study investigated the influence of the AHR-ligand 3-methylcholanthrene (3-MC) on ER-mediated signaling in mammary gland tissue of ovariectomized (ovx) female rats.

Methods: After 14 days of hormonal decline, ovx rats were treated for 3 days with 4 µg/kg 17β-estradiol (E2), 15 mg/kg 8-Prenylnaringenin (8-PN), 15 mg/kg 3-MC or a combination (E2+3-MC, 8-PN+3-MC). Whole mount preparations of the mammary gland were used to count terminal end buds (TEBs). Protein expression studies (Immunohistochemistry, Immunofluorescence), a cDNA microarray, pathway analyses and *qPCR*s were performed to evaluate the interaction between AHR- and ER-mediated signaling pathways.

Results: E2 treatment increased the number of TEBs, protein levels of Ki-67 and progesterone receptor (PR) and changed the expression level of 325 genes more than 1.5 fold. 3-MC treatment alone had marginal impact on gene or protein expression. However, in the situation of a co-treatment of the rats, 3-MC strongly inhibited E2 induced TEB development, protein expression and the expression of nearly half of E2 induced genes. This inhibitory effect of 3-MC was partially mirrored as 8-PN was used as an ER-ligand. The anti-estrogenicity of ligand-activated AHR was at least partly due to decreased protein levels of ERα in ductal epithelial cells.

Conclusion: Our data show transcriptome wide anti-estrogenic properties of ligand-activated AHR on ER-mediated processes in the mammary gland, thereby contributing to evaluate the chemopreventive and endocrine disrupting potential of AHR-ligands.

Introduction

To evaluate the influence of endocrine disrupting natural compounds or selective estrogen receptor modulators, it is crucial to understand the underlying mechanisms. This study was designed to investigate the impact of ligand-activated Aryl hydrocarbon receptor (AHR) on ligand-activated Estrogen receptor (ER) signaling pathways in the rodent mammary gland.

The AHR is a ligand dependent transcription factor, that belongs to the basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) family (Hankinson 1995; Matsumura and Vogel 2006; Mimura and Fujii-Kuriyama 2003; Poellinger 2000). The best-characterized ligands for the AHR are planar hydrophobic molecules including many environmental contaminants, such as halogenated hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) (reviewed in Denison et al. 2002). After ligand binding, AHR translocates to the nucleus, dimerizes with its partner protein, aryl hydrocarbon receptor nuclear translocator (ARNT), and the AHR/ARNT-heterodimer binds to xenobiotic response elements (XREs), inducing gene expression of drug-metabolizing enzymes such as CYP1A1 and CYP1B1 (Beischlag et al. 2008; Larsen et al. 1998; Whitlock et al. 1996). In addition to its major role in xenobiotic metabolism, the AHR is involved in regulation of cell proliferation, apoptosis, adipose differentiation, tumor suppressor functions, immune cell differentiation and reproductive function (Hernandez-Ochoa et al. 2009; Kerkvliet 2002; Puga et al. 2009). The physiological consequences on individual tissues have not yet been fully elucidated. In the mammary gland the AHR is dispensable for development (Le Provost et al. 2002), but the receptor appears to be an important factor in the regulation of proliferation, since in AHR null mice the number of terminal end buds (TEBs) is reduced by 50% during estrous-stimulated growth (Hushka et al. 1998). These AHR-mediated responses in the mammary gland are probably due to cross-talk between AHR and steroid receptors, including

ERα, ERβ and androgen receptor (AR) as has been reported in cell lines and the mouse uterus (Beischlag and Perdew 2005; Matthews et al. 2005; Ohtake et al. 2003; Ohtake et al. 2007; Ohtake et al. 2008). How ligand-activated AHR influences ER signaling on a molecular level remains unclear. Several different mechanisms have been proposed, including direct binding to ERs, the competition of AHR/ARNT-complex and ER for common cofactors (p300, p160, CBP), induction of CYPs by activated AHR leading to enhanced E2 metabolism, or the proteasomal degradation of ER triggered by liganded AHR (for overview see Shanle and Xu 2011; Swedenborg and Pongratz 2010).

PAHs are widespread AHR ligands that mainly form in the process of incomplete combustion and are therefore abundant constituents of exhaust fumes from cars and factories as well as of cigarette smoke. Because the synthetic PAH 3-methylcholanthrene (3-MC) has a carcinogenic potential that is higher than that of most other PAHs like benzo[a]pyrene, it is frequently employed to induce tumor formation in mice (Chen et al. 2015; Rymaszewski et al. 2014). Nevertheless it is frequently used in studies investigating AHR signaling because it is known to be a strong activator of AHR (Riddick et al. 1994). In addition 3-MC is readily metabolized, further decreasing its effectiveness regarding the activation of AHR *in vivo*, while other AHR ligands like TCDD are extremely persistant (Riddick et al. 1994). This makes 3-MC a suitable model to study effects of PAHs on ER signaling. In this manuscript we describe the *in vivo* effects of E2, 3-MC and E2 plus 3-MC on transcriptional and translational regulation of mRNA or protein levels to develop mechanistic understanding of the interaction between AHR- and ER-mediated signaling pathways in the rat mammary gland in a physiological context. Using a transcriptome-based approach we have characterized ER- and AHR-regulated genes and the overall expression change triggered by the respective receptors. Additionally, we treated rats

with either the potent phytoestrogen 8-prenylnaringenin (8-PN) (Milligan et al. 1999; Zierau et al. 2008) alone or in co-treatment with 3-MC.

Methods

Substances

17β-estradiol (E2) and 3-methylcholanthrene (3-MC) were purchased from Sigma-Aldrich. 8-prenylnaringenin (8-PN) was synthesized from naringenin as described previously (Gester et al. 2001). The purity of the compound was assessed to be > 99% by gas chromatography and HPLC. 8-PN was used as a racemic mixture.

Animals

Young adult female Lewis rats (200 g) were maintained under controlled conditions of temperature (20 ± 1 °C, relative humidity 50-80%) and illumination (12 h light, 12 h dark). Animals were housed in open cages (Tecniplast) with four to six animals per cage. For bedding a mixture of Lignocell wood chips and Rehofix corncob granules (JRS Rettenmaier & Söhne) was used. All rats had free access to standard phytoestrogen-free rat diet (Teklad Global Diet) and water. Animals used in this study were treated humanely and with regard for the alleviation of suffering. The experimental design complied with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines published by the National Centre for the Replacement, Refinement and Reduction of Animals in Research (https://www.nc3rs.org.uk/). All procedures were approved by and carried out according to the institutional and state Animal Care and Use Committee guidelines, regulated by the German federal law for animal welfare.

Three day in vivo assay

Approximately ten week old rats were bilaterally ovariectomized (ovx). 14 days after ovx, rats were treated subcutaneously at 0, 24 and 48 h with the test compounds (E2, 3-MC, E2+3-MC, 8-PN and 8-PN+3-MC) in the same room the animals were housed. The treatment of the animals was carried out 2 h after the start of the light period of the day. E2 was administered at 4 µg/kg body weight (BW)/day, 3-MC and 8-PN at 15 mg/kg BW/day. The combination doses for E2+3-MC were 4 µg/kg+15 mg/kg, respectively, while the doses for 8-PN+3-MC were 15 mg/kg+15 mg/kg, respectively. The substances were dissolved in a DMSO/castor oil mixture which served also as the negative control (referred to as the vehicle). The animals were randomly selected for treatment and vehicle groups (E2 group n=13, vehicle group n =11, other treatment groups n=6). Necropsies were performed 24 h after the final treatment using CO_2 inhalation subsequent to a light O_2/CO_2 anaesthesia. The 4th right mammary gland was whole mounted, while the 4th left mammary gland was excised and fixed in 4% paraformaldehyde for paraffin embedding. The 2nd, 3rd and 5th mammary glands from both sides were snap frozen in liquid nitrogen for RNA preparation.

RNA preparation

Total cytoplasmic RNA was extracted from the mammary gland tissue by the standard TRIzol® method (Life Technologies). The quality and the concentrations of the preparations of total RNA were determined using the NanoDrop (Thermo Fisher Scientific) and Agilent Bioanalyzer (Agilent Technologies).

mRNA microarray analyses

Total RNA (100 ng) from 3 rats per treatment group (vehicle, E2, 3-MC, E2+3-MC) and deemed to be of good quality (RIN $\geq$ 7) was processed according to the standard Affymetrix Whole

Transcript Sense Target labeling protocol. The fragmented biotin-labeled cDNA was hybridized for 16 h to Affymetrix Gene 1.0 ST arrays and scanned on an Affymetrix Scanner 3000 7G using AGCC software. The resulting CEL files were analyzed for quality using Affymetrix Expression Console software, and imported into GeneSpring GX11.5 (Agilent Technologies). The data was quantile-normalized using PLIER and baseline transformed to the median of the control samples. The probe sets were further filtered to exclude the bottom 20th percentile across all samples. The resulting entity list was subjected to an unpaired T-test with Benjamini-Hochberg False Discovery rate correction and a 1.5 fold filter to identify differentially expressed transcripts between conditions at a p-value <0.05. Pathway analysis and functional annotation clustering of E2 regulated genes (p-value <0.05) were performed using DAVID 6.7 (Huang et al. 2009). All data have been deposited in the Gene Expression Omnibus (GEO, accession number GSE64636, Edgar et al. 2002).

qPCR validation of microarray data

Changes in mRNA identified by microarray were validated using 5-6 independent biological replicates. Reverse transcription PCR reactions were performed with 1.5 µg total RNA using Taqman® Reverse Transcription Reagents (Applied Biosystems) to synthesize cDNA for mRNA expression analysis. Quantitative PCR (*qPCR*) analyses were performed following the protocol described previously (Wang W et al. 2011), and analyzed using the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems): first denaturing cycle at 95^{0}C for 3 min, then 95^{0}C for 15 s, 60^{0}C for 10 sec and 72^{0}C for 20 s, repeated for 40 cycles. Fluorescence was quantified at the end of the 72°C annealing step and the amplicon specificity was confirmed by a melting curve analysis (60-95°C). Primer sequences are summarized in Table S1 (see supplemental material). The relative mRNA amounts of target genes were calculated after normalization to an

endogenous reference gene (ribosomal protein S18). Results were expressed as relative amounts of mRNA compared to the vehicle control animals using the $2^{-\Delta\Delta CT}$ method (Pfaffl 2001).

Mammary gland whole mount preparations

Whole mount preparations were performed as previously described (de Assis et al. 2010). After staining with Carmine Alum (0.2% carmine, 0,5% aluminium potassium sulfate), the samples were coated with polyacrylate, placed in a water filled petri dish and viewed under a Stereomicroscope (Stemi DRC, Carl Zeiss AG) to count the number of terminal end buds (TEB). From every treatment group the TEBs of at least 6 animals per group were counted.

Immunohistochemistry

Dissected mammary tissue was fixed in 4% paraformaldehyde, embedded in paraffin and sectioned at 3 µm. Antigen retrieval was performed with 10 mM Tris-EDTA, pH 9 at 60 °C overnight. Proteins of interest were stained using the IHC Select™ Immunoperoxidase Secondary Detection System (Millipore) according to the manufacturer`s protocol. The primary antibodies anti-progesterone receptor (PR) (1:350, Thermo Fisher Scientific), anti-Ki-67, a cell proliferation marker (1:150, Abcam) and anti-AHR (1:250, Abcam) were diluted in 5% filtered skim milk powder in PBS and incubated for 1 h at room temperature in a humidified chamber. Sections were counterstained with haematoxylin (Carl Roth). Visualization was done using a Keyence BZ-8100E (Keyence) microscope. For the quantification of Ki-67 and PR positive cells, at least 500 luminal epithelial cells from each of 5 or more animals per group were analyzed and scored using ImageJ 1.47. AHR expression was quantified by measuring the DAB staining intensity of luminal epithelial cells and normalized in relation to the negative control group using ImageJ 1.47. For fluorescent ERα staining, sections were blocked with 5% BSA/TBS + 0.1% Triton X-100 for 1 h at room temperature in a humidified chamber. Primary anti-ERα antibody

(1:250, Abcam) was diluted in 2.5% BSA/TBS and the tissue sections were incubated overnight at room temperature. After washing with TBS, sections were incubated with CruzFluor™ 488 conjugated goat-anti rabbit secondary antibody (1:200 in 2.5% BSA/TBS, Santa Cruz Biotechnology) for 1 h. Sections were mounted with Mowiol (Carl Roth) and counterstained with diamidino-2-phenylindole (DAPI, 1:250, Santa Cruz). An Axiovert 100 microscope with a Colibri.2 LED illumination system (Carl Zeiss AG) was used to visualize the fluorescence stained mammary gland epithelial cells. Magnification and exposure time were set identically to identify fluorescence intensity differences between treatment groups. The fluorescence intensity per duct was measured and normalized in relation to the negative control group using ImageJ 1.47.

Statistical analysis

The data are presented as arithmetic mean ± standard deviation (SD) for immunohistochemistry and *qPCR* data or as median with percentiles (25% to 75%) for TEB count and immunofluorescence. Statistical analysis included one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test to determine significant differences for the *qPCR* results, TEB count, immunohistochemistry and immunofluorescence in the mammary gland. The results were considered to be statistically significant at $p \leq 0.05$.

Results

The affinity of 3-MC to the AHR is somewhat lower than that of the prototypical ligand TCDD. Its IC_{50} in a competitive ligand binding assay is about 4fold higher than that of TCDD while the concentration necessary to induce Cyp1a1 mRNA expression is between 10 and 100fold higher (Riddick et al. 1994). We therefore opted for a much higher treatment dose of 15mg 3-MC per kg body weight per day than what is usually used for treatment with TCDD. This dose is similar

to that used in many other studies of 3-MC as an AHR ligand (Deb and Bandiera 2010; Göttlicher and Cikryt 1987; Kondraganti et al. 2005).

Effect of 3-MC on terminal end bud development

To determine the nature of the interaction between the ligand-activated AHR and ER pathways, the effect of treating ovx rats for 3 days with E2 and 3-MC, alone or in combination, on the TEB numbers in whole mount preparations of the mammary gland was assessed (Figure 1A). Quantitation of these data is presented in Figure 1B. Estradiol significantly increased the number of TEBs (46.2±18.2) when compared to vehicle treated controls (8.1±3.5). Differences in TEB numbers were also significant between 3-MC (11.8±2.9) and vehicle treated controls, even though to a far lesser extent. Co-treatment with E2 and 3-MC significantly reduced the number of TEBs compared to E2 alone (29.2±9.5), although the number of TEBs remained higher than in the 3-MC treated animals. These data suggest that the liganded AHR pathway attenuates, but does not completely block E2 signaling mediated by the ER.

Protein expression of ERα and AHR

To determine whether the effect of 3-MC on E2 stimulation of TEB formation was the result of changing receptor expression levels, the status of ERα and AHR in the mammary gland was assessed. The relative mRNA levels of the ER (*Esr1, Esr2*) and *Ahr* were not significantly different in any of the treatment groups as measured by *qPCR* (see supplemental material, Figure S1). The steady state protein levels of ERα were significantly increased in the ductal epithelial cells after treatment with E2 alone compared to the vehicle control group (Figure 2A, B). In contrast, 3-MC alone caused a significant decrease of relative fluorescence intensity compared to the vehicle control, and 3-MC further abrogated the effect of E2 on ERα expression when co-administered (Figure 2B). This suggests that 3-MC exerts a strong anti-estrogenic effect by

down-regulating ERα expression. In contrast, neither 3-MC nor E2 induced any significant changes in the AHR protein in ductal epithelial cells of the mammary glands, either alone or in combination (Figure 2C, vehicle 100±17.7% relative fluorescence intensity, E2 98.9±10.1%, 3-MC 96.8±15.2%, E2+3-MC 99.3±7.4%).

Effect of 3-MC on Ki-67 and PR expression

To investigate the cellular mechanisms responsible for the anti-estrogenic effects of 3-MC we examined the expression of the proliferation marker Ki-67 and PR, a classical E2-regulated target in the mammary ductal epithelium (Haslam and Shyamala 1979). As seen in Figure 3, Panels A and C, the percentage of ductal epithelial cells expressing Ki-67 increased from 1% in the vehicle treated control animals to 44.9% after three days E2 treatment. 3-MC alone did not increase Ki-67 expression but completely attenuated E2-induced expression of Ki-67 (4%). Similarly, treatment with E2 increased the number of PR positive epithelial ductal cells to 53%. However, while co-administration of 3-MC reduced this effect significantly to 40% (Figure 3D), it did not completely abrogate the E2-induced increase in PR levels. These data suggest that while many of the effects of 3-MC on E2 regulation of tissue growth may be attributable to its effects on ER levels, some of the physiological effects of ER are more sensitive to 3-MC than others.

Genome wide transcriptome analysis

The effects of E2, 3-MC and E2+3-MC on gene expression in the mammary gland were assessed by cDNA-microarray. A 1.5 fold change filter was used to compare the expression levels resulting from the respective treatments (3-MC, E2, E2+3-MC) to the vehicle controls.

Treatment with E2 differentially modulated the expression of 325 entities (Figure 4A), including 272 entities (84%) that were upregulated and 54 (17%) that were downregulated compared with

the vehicle treated controls. The expression change of the 15 most upregulated genes by E2 ranged from 16 fold for beta-casein (*Csn2*) to 4 fold for kinesin family member 11 (*Kif11*) (Figure 4B). While a number of genes were downregulated by E2, the magnitude of the downregulation is less dramatic, ranging from -4 fold to -2 fold for the 15 most downregulated genes. Pathway analyses of E2-regulated genes identified 5 predominant pathways (cell cycle, cell adhesion molecules, p53 signaling pathway, pyruvate metabolism, insulin signaling pathway, $p \leq 0.05$, see supplemental material, Table S2). Genes involved in the control of cell division were exclusively upregulated by E2 (Table S2). The regulatory role of these E2 upregulated genes in proliferative processes was confirmed in a functional annotation clustering (see supplemental material, Figure S2). 3-MC alone modulated a total of 30 differentially expressed entities (Figure 4A), of which 25 were up- and 5 downregulated. However only 2 of these entities (*Lefty1* and the mRNA for the uncharacterized protein LOC502684) were solely regulated by 3-MC, both close to the 1.5 cut off, indicating that under these experimental conditions 3-MC alone had minimal effects on mammary gland gene expression. Moreover 3-MC substantially attenuated E2 mediated signaling (Figure 4C) and dampened the inductive and suppressive effects of E2 on both, up- and downregulated gene expression of 132 of the 325 genes modulated by E2 (Figure 4D). These data demonstrate that 3-MC has a significant global antagonist action on the biological activity of E2 in the mammary gland of ovx rats.

qPCR validation of selected genes

To validate the regulation of differentially regulated genes identified in the microarray, selected genes associated with the major ontologies affected by E2 (cell cycle, cell adhesion and p53-mediated signaling) were analyzed by *qPCR*. E2 had a significant effect on the expression of genes associated with cell division (*Top2a*, *Mki67*, *Ccnb1*, *Ccnb2*, Figure 5A) and cytokinesis

(*Kif11*, *Kif2c*, *Kif18a*, Figure 5B). While 3MC alone had an effect on *Top2a*, *Ccnb1* and *Kif2c*, mRNA expression, which was far less pronounced than for E2 alone, it significantly attenuated the E2 upregulation of *Top2a*, *Ccnb2* and *Mki67*. We also studied the effects of E2 and 3-MC on the expression of several E2-regulated genes responsible for differentiated functions of the mammary gland, including genes associated with the synthesis and secretion of milk (*Areg*, *Csn2*, Pgr, *Wap*, *Aqp5* and *Prlr*), which were upregulated by E2 in the microarray, and *Retn,* which was repressed by E2 (Figure 5C and D). While 3-MC alone had a small, but statistically significant effect on *Areg*, *Pgr* and *Aqp5* expression, it significantly reduced the upregulation of E2 for *Pgr* and *Wap*.

Influence of 3-MC on phytoestrogen 8-PN-induced effects

To determine whether the effects of 3-MC are ER-ligand dependent and whether the abrogation of estrogenic signaling by 3-MC is important in natural compound endocrine disruptor interactions, rats were treated with the phytoestrogen 8PN alone or in combination with 3-MC, recapitulating the experiments described above. 8-PN significantly increased the number of TEBs, although it is clear that in this bioassay system 8-PN is significantly less estrogenic than E2, inducing 18.7±4.6 versus 46.2±18.2 TEBs per gland (Figure 6A, Figure 1B). The inductive effect of 8-PN was inhibited by 50% by co-treatment with 3-MC (Figure 6A). The increase in TEBs in response to 8-PN was reflected in the increase in the percent of Ki-67 positive cells, which was substantially lower compared to E2 (8.1±4.1% versus 44.9±14.7%), confirming the weaker estrogenic potency of 8-PN. Co-treatment with 3-MC effectively blocked the 8-PN induced increase in proliferating cells, but statistical significance was not achieved (p=0.05891, Figure 6B). To investigate the effects of 8-PN alone and in co-treatment with 3-MC on gene expression, we chose genes highly regulated by E2 as identified above (*Areg, Csn2, Pgr*), as well

as genes associated with proliferation (*Kif*, *Mki67*, Figure 6C).While 8-PN weakly induced the expression of some of the E2-target genes (*Kif11*, *Kif2c*, *Kif18a*, *Csn2*), a significant counteracting effect of 3-MC on 8-PN-induced gene expression could be observed for *Csn2, Kif18a* and *Kif11* in which the relative expression of the mRNA was significantly abrogated after co-treatment . However, some of the gene expression patterns induced by 8-PN were different than the ones induced by E2, as 8-PN decreased *Mki67* expression and had no significant effect on *Areg* or *Pgr* expression.

Discussion

Interaction of xenobiotics with the hormonal system in the mammary gland is of great relevance regarding cancer risks and other syndromes whose etiologies are associated with hormonal interferences. In this manuscript we have studied the cross-talk between ER- and AHR-mediated signaling processes in the female rat mammary gland by using the ovariectomized rat mammary gland model system. This provides a very sensitive model system to examine physiological effects of E2, and the interaction between E2 and environmental aryl hydrocarbon receptor ligands such as TCDD and 3-MC.

The data presented in this manuscript is in line with the large body of literature demonstrating antiestrogenic effects of AHR ligands in cell culture (Gierthy et al. 1987; Safe and Krishnan 1995; Ueng et al. 2004; Wang F et al. 1998; Wormke et al. 2000). We could demonstrate that 3-MC has inhibitory effects on the expression of a significant proportion of E2-modulated genes and has pronounced effects on the physiological responses to E2. Within the last 15 years there have been several reports of inhibitory effects of TCDD on mammary gland development in animals (Fenton et al. 2002; Vorderstrasse et al. 2004) as well as in humans (Den Hond et al. 2002). In addition animal studies (Collins et al. 2009; Vorderstrasse et al. 2004) and experiments

using primary mammary epithelial cells and HC-11 cells (Basham et al. 2015) suggest that AHR activation has a negative impact on milk production resulting in the inability of mice to nutritionally support their offspring (Vorderstrasse et al. 2004). The inhibition of E2 induced development of terminal end buds, proliferation and upregulation of cell cycle and proliferation related pathways we have observed in response to 3-MC treatment suggests that these effects are independent of the specific AHR ligand and could therefore also be caused by the much more abundant PAHs. Furthermore our data suggests that the adverse effects of AHR ligands on mammary gland development are probably based on their ER antagonistic properties. These data also offer an explanation for the observed decrease of mammary tumors in female rats after long term TCDD treatment (Kociba et al. 1978), and the well-known inhibitory properties of exogenous AHR-ligands on rodent mammary gland tumors and metastasis development (Lubet et al. 2011; Safe 2001; Wang T et al. 2011). Given the similarity between the development and carcinogenesis of the rodent and human mammary glands (Rudel et al. 2011), the anti-estrogenic effects of the AHR-mediated signaling may at least partially explain the increased overall survival and distant metastasis free survival in hormone dependent ER-positive breast cancers in human patients with elevated AHR expression (O'Donnell et al. 2014; Saito et al. 2013).

There are several mechanisms that might explain these ER antagonistic effects. Many AHR ligands, among them 3-MC, have been shown to directly interact with ERs (Abdelrahim et al. 2006; Liu et al. 2006). In the case of 3-MC this interaction is very weak but measurable. This affinity to both ERs may lead to an activation of ERs in some tissues but antagonistic effects in others. Notably in mammary gland derived HC11 cells 3-MC acts as an AHR independent ERα antagonist (Swedenborg et al. 2008) and this is therefore also likely to be the case in the mammary gland. On the other hand TCDD displays similar antiestrogenic properties even

though its affinity to ERα and ERβ is even lower and it is usually tested at much lower concentrations due to its persistency. In this study only the expression changes of a subset of the E2 regulated genes are blocked by 3-MC treatment. It is therefore likely that additional mechanisms are involved in mediating the antiestrogenic response of AHR ligands in general and 3-MC specifically. Firstly, AHR-ligands alter hepatic E2 metabolism in female rats through the activation of several hepatic P450 cytochromes, increasing conversion to 2-hydroxyestradiol and 7α-hydroxyestradiol and by that decreasing the bioavailability of E2 (Suchar et al. 1996). Secondly, the AHR has been identified as a component of a cullin 4B ubiquitin ligase complex ($CUL4B^{AHR}$) (Ohtake et al. 2007), and in the rat uterus activation of the AHR by 3-MC and other ligands of the receptor results in ubiquitinylation and proteasomal degradation of the ER and other steroid receptors (Wormke et al. 2003). The decrease in the level of the ligand (due to metabolism) and/or the receptor (due to degradation) would be anticipated to have global effects on E2-dependent gene expression in the mammary gland. While 3-MC has significant inhibitory effects on E2-mediated modulation of many genes in the mammary gland (132/325, 41%), a great proportion (193/325, 59%) of the E2-responsive genes are not affected by 3-MC (Figure 4). This indicates that the effects of 3-MC on mammary gland physiology and gene expression are not solely (or even primarily) due to its effects on hepatic metabolism or the stability of the estrogen receptor. This is also supported by the observed impact of 3-MC on the estrogenic actions of 8-PN, a natural occurring, plant derived ER ligand (Kitaoka et al. 1998; Helle et al. 2014; Milligan et al. 1999). Even though 8-PN is a weak estrogen and metabolized differently in the liver (Guo et al. 2006; Keiler et al. 2015), the effects of 3-MC on 8-PN-mediated gene expression mirror partially effects seen with E2. Importantly, co-treatment with 3-MC also

counteracts some of the effects of 8-PN suggesting that the inhibitory effects of 3-MC are independent of which ligand is bound to the ER.

Conclusions

Our data give a detailed account of the *in vivo* effects of E2 and 3-MC on gene expression and development of mammary glands and demonstrate that AHR ligands like 3-MC are antiestrogenic with regard to many E2 regulated genes in the mammary gland. Almost no E2 independent effects of 3-MC on gene expression could be observed. This indicates that interaction with ERs is the predominant mode of action of AHR in the mammary gland. This helps to explain the adverse effects of AHR ligands on mammary gland development and underlines the potential of AHR as a breast cancer therapy target. We could identify degradation of the ERα as another putative mechanism by which 3-MC and possibly other PAHs inhibit ER signaling *in vivo*. In addition, changes in gene expression caused by the phytoestrogen 8-PN also were reduced following 3-MC treatment, but further studies will be required to assess the importance of the interactions between ERs and AHR with regard to different endogenous, exogenous, nutrition-derived and synthetic ER ligands *in vivo*.

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Figure Legends

Figure 1. Effect of E2 and 3-MC, alone and in combination, on terminal end bud (TEB) formation in ovariectomized (ovx) rats.

Ovx rats were treated with E2, 3-MC, E2+3-MC or vehicle control for three days. Mammary gland whole mounts were prepared and stained as described in methods.

Panel A: Representative TEB formation in mammary whole mounts from each treatment group. Arrows highlight TEBs. Magnification scale: 500 μm.

Panel B: Quantification of the effects of E2 and 3-MC, alone and in combination, on TEBs formation. Diamonds represent individual animals; box show 25^{th} to 75^{th} percentile, while the horizontal line indicates the median value. The whiskers mark the highest and the lowest measured value partitioned by the median. * significant differences ($p<0.05$)from vehicle control (one way ANOVA). # significant differences from E2 treatment alone (one way ANOVA).

Figure 2. Effect of E2 and 3-MC, alone and in combination, on ERα and AHR receptor status in mammary glands from ovariectomized (ovx) rats.

Ovx rats were treated with E2, 3-MC, E2+3-MC or vehicle control for three days.

Panel A: Representative expression of ERα in mammary glands from each treatment group. Tissue sections were prepared for immunofluorescence microscopy as described in methods. Magnification scale: 30 μm.

Panel B: Quantification of the effects of E2 and 3-MC, alone and in combination, on ERα expression in the mammary glands of 6 animals per treatment group. Fluorescence-intensity per duct was measured as described in methods and normalized to the vehicle control. Diamonds represent individual ducts; box show 25^{th} and 75^{th} percentile, while the horizontal line indicates the median value. The whiskers mark the standard deviation for each treatment group. * significant differences ($p<0.05$) from vehicle control (one way ANOVA). # significant differences from E2 treatment alone (one way ANOVA). + significant differences from 3-MC treatment alone (one way ANOVA).

Panel C: Representative immunohistochemical staining for AHR in mammary gland ducts labeled with an antibody against AHR for each treatment group. Paraffin embedded sections were prepared for immunohistochemistry using 3,3'-Diaminobenzidine (DAB) as described in methods. Magnification scale: 50 μm.

Figure 3. Ki-67 and PR protein expression in the mammary gland

Ovx rats were treated with E2, 3-MC, E2+3-MC or vehicle control for three days. Paraffin embedded sections were prepared for immunohistochemistry using 3,3'-Diaminobenzidine (DAB) as described in methods. Paraffin embedded sections of rat mammary glands from each treatment group were stained with DAB using antibodies against Ki-67 (Panel A) or PR (Panel B) and counterstained with haematoxylin. Magnification scale: 50 μm.

Panel C, D: Quantification of the effects of E2 and 3-MC, alone and in combination, on Ki-67 and PR protein expression. The number of positively stained epithelial cells were counted and normalized to the total number of epithelial cells. At least 500 cells were evaluated per animal, 5 to 6 animals were used per treatment group. Data are presented as arithmetic mean ± SD. * significant differences ($p<0.05$) from vehicle control (one way ANOVA). # significant differences from E2 treatment alone (one way ANOVA).

Figure 4. Genome wide transcriptome analysis of effects of E2 and 3-MC, alone and in combination, in mammary glands from ovx rats.

Ovx rats were treated for three days with E2, 3-MC, E2+3-MC or vehicle control. RNA was extracted from mammary glands from each treatment group and global gene expression changes were analyzed by microarray as described in methods. Three independent biological replicates were interrogated for each treatment group.

Panel A: Venn diagram illustrating the number of entities modulated by each treatment and overlap in target genes between treatment groups. Fold change (FC) ≥ 1.5 compared to the vehicle control.

Panel B: Fold Change of all entities modulated by entities from the mammary gland of E2 or E2+3-MC treated rats compared to vehicle treated control.

Panel C: Comparison of the FC of 15 most up- and downregulated genes by E2 and E2+3-MC treatment.

Panel D: Interaction analysis between E2 and 3-MC mediated gene expression.

The FC of entities regulated by E2+3-MC and E2 are compared. The relative expression values of the E2 treatment group were set to 0. Fold change (FC) ≥ 1.5 compared to the E2 group.

Figure 5. Expression of selected genes in mammary glands in ovariectomized (ovx) rats in response to E2 and 3-MC, alone and in combination.

Ovx rats were treated for three days with E2, 3-MC, E2+3-MC or vehicle control. RNA was extracted from mammary glands and expression of individual genes was assessed using *qPCR* as described in methods. Panel A: genes associated with cell cycle and proliferation; Panel B: genes associated with cytokinesis; Panel C: classical E2-responsive genes; Panel D: genes associated with differentiated mammary gland function. Data are presented as fold change, mean ± SD for each treatment group relative to vehicle treated controls. Five to six animals per treatment group were analyzed. * significant differences ($p<0.05$) from vehicle control (one way ANOVA). # significant differences from E2 treatment alone (one way ANOVA).

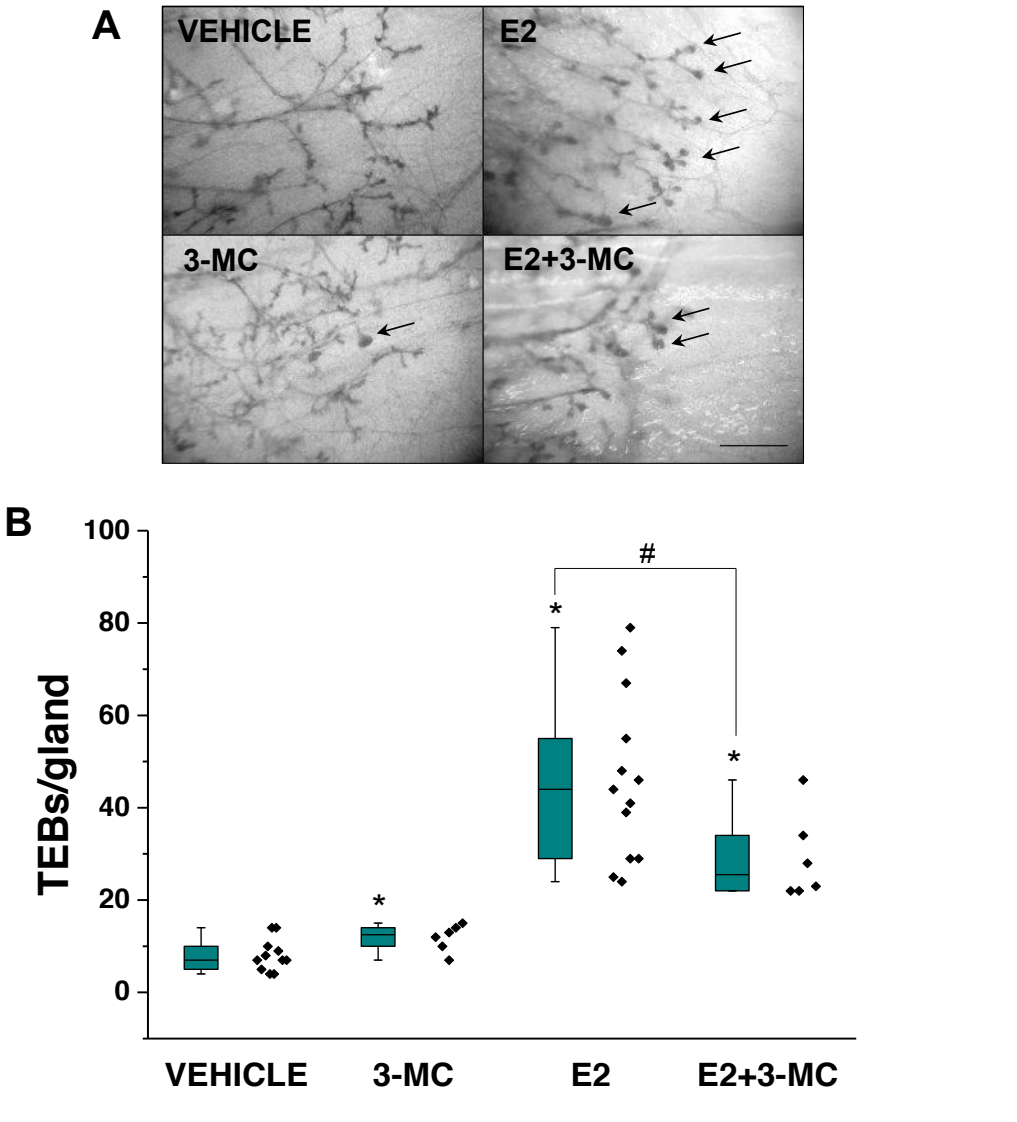
Figure 6. Effect of 8-PN and 3-MC, alone and in combination, on TEB number, Ki-67 staining and mRNA expression in mammary glands from ovariectomized (ovx) rats.

Ovx rats were treated for 48 h with 3-MC, 8-PN, 8-PN+3-MC or vehicle control as described in methods.

Panel A: Quantitation of the effects of 8-PN and 3-MC, alone and in combination, on TEBs formation in mammary whole mounts. Mammary gland whole mounts were prepared, stained and quantitated as described in methods. Diamonds represent individual animals; box show 25^{th} and 75^{th} percentile, while the horizontal line indicates the median value. The whiskers mark the highest and the lowest measured value for each treatment group. * significant differences ($p<0.05$) from vehicle control (one way ANOVA). # significant differences from 8-PN treatment alone (one way ANOVA).

Panel B: Ki-67 expression in mammary glands from ovx rats treated with 8-PN or 3-MC alone or in combination. Paraffin embedded sections of rat mammary glands from each treatment group were stained with DAB using antibodies against Ki-67 and counterstained with hematoxylin. The number of positively stained epithelial cells were counted and normalized to the total number of epithelial cells. At least 500 cells were evaluated per animal, 5 to 6 animals were used per treatment group. Data are presented as arithmetic mean ± SD. * significant differences ($p<0.05$) from vehicle control (one way ANOVA). # significant differences from 8-PN treatment alone (one way ANOVA).

Panel C: *qPCR* analysis of selected genes in mammary glands from ovx rats treated with 8-PN or 3-MC alone or in combination. Results are presented as mean ± SD, n=5-6; * significant differences ($p<0.05$) from vehicle control (one way ANOVA). # significant differences from 8-PN treatment alone (one way ANOVA).


A
VEHICLE
E2
3-MC
E2+3-MC
B
TEBs/gland
100
80
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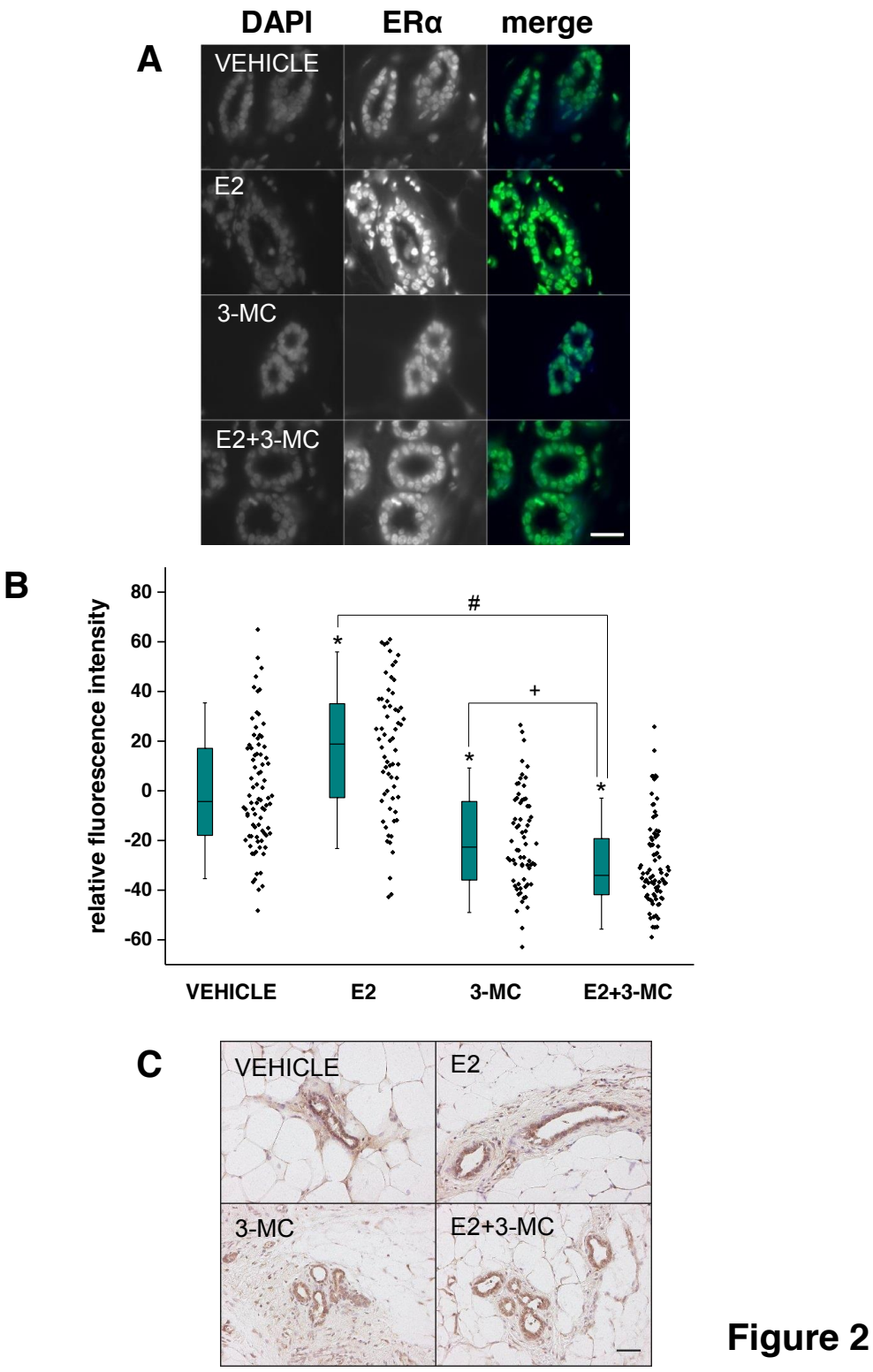
VEHICLE
3-MC
E2
E2+3-MC

Figure 1


A
DAPI
ERα
merge
VEHICLE
E2
3-MC
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B
relative fluorescence intensity
80
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40
20
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-20
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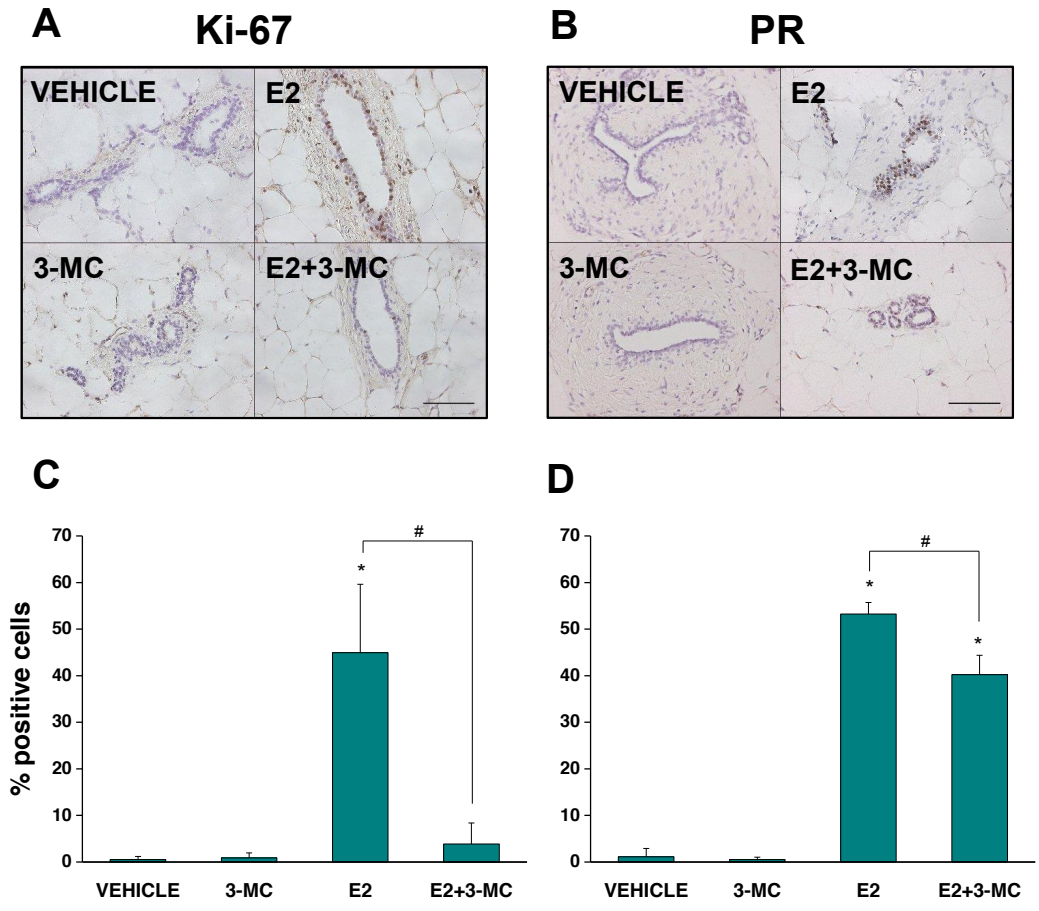
E2+3-MC

Figure 2


A
Ki-67
VEHICLE
E2
3-MC
E2+3-MC
B
PR
VEHICLE
E2
3-MC
E2+3-MC
C
% positive cells
70
60
50
40
30
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E2+3-MC

Figure 3

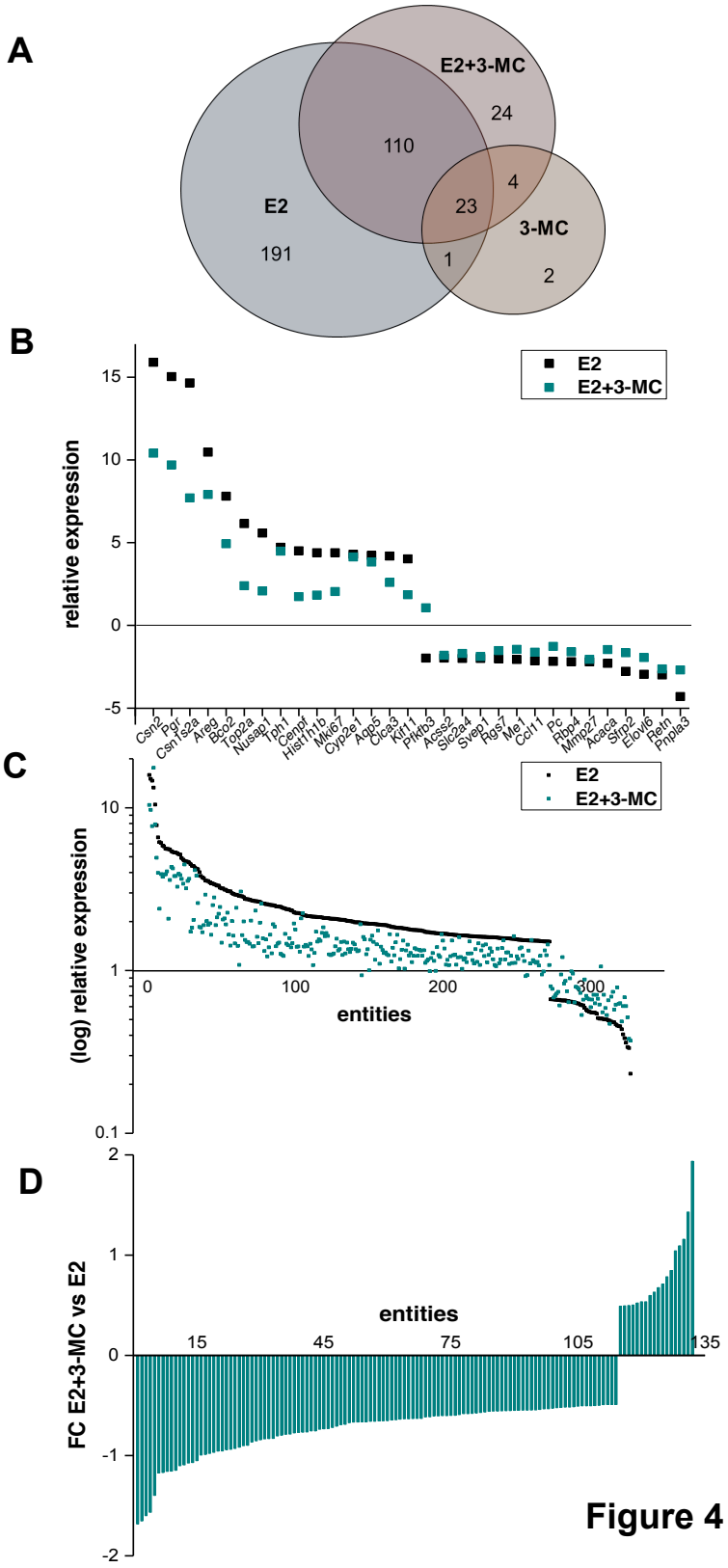
A
E2+3-MC
24
110
4
E2
23
3-MC
191
1
2
B
E2
E2+3-MC
relative expression
15
10
5
0
-5
Csn2
Pgr
Csn1s2a
Areg
Bcp2
Top2a
Nusap1
Tph1
Cenpf
Hist1h1b
Mki67
Cyp2e1
Aqp5
Clca3
Kif11
Pfkfb3
Acss2
Slc2a4
Svep1
Rgs7
Me1
Ccl11
Pc
Rbp4
Mmp27
Acaca
Sfrp2
Elovl6
Retn
Pnpla3
C
E2
E2+3-MC
(log) relative expression
10
1
0.1
0
100
200
300
entities
D
FC E2+3-MC vs E2
2
1
0
-1
-2
entities
15
45
75
105
135

Figure 4

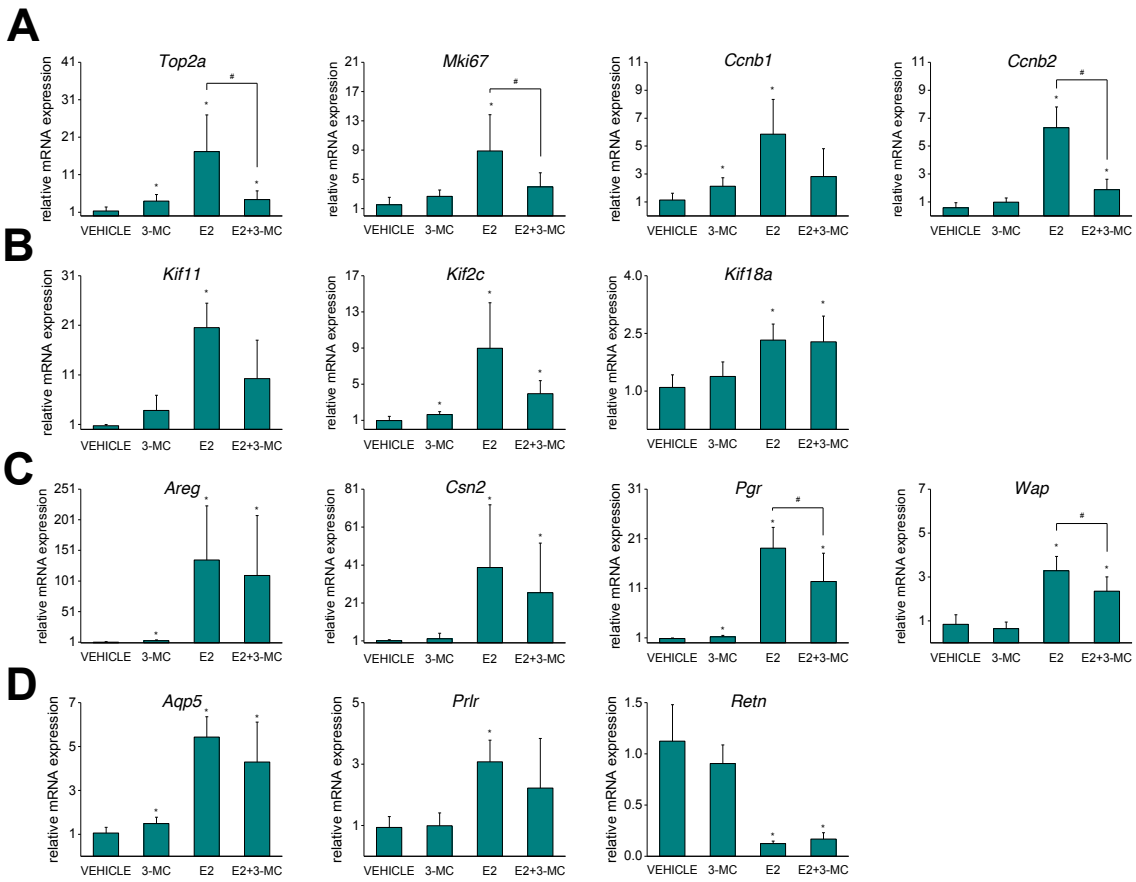
A
Top2a
Mki67
Ccnb1
Ccnb2
B
Kif11
Kif2c
Kif18a
C
Areg
Csn2
Pgr
Wap
D
Aqp5
Prlr
Retn
relative mRNA expression
VEHICLE
3-MC
E2
E2+3-MC

Figure 5

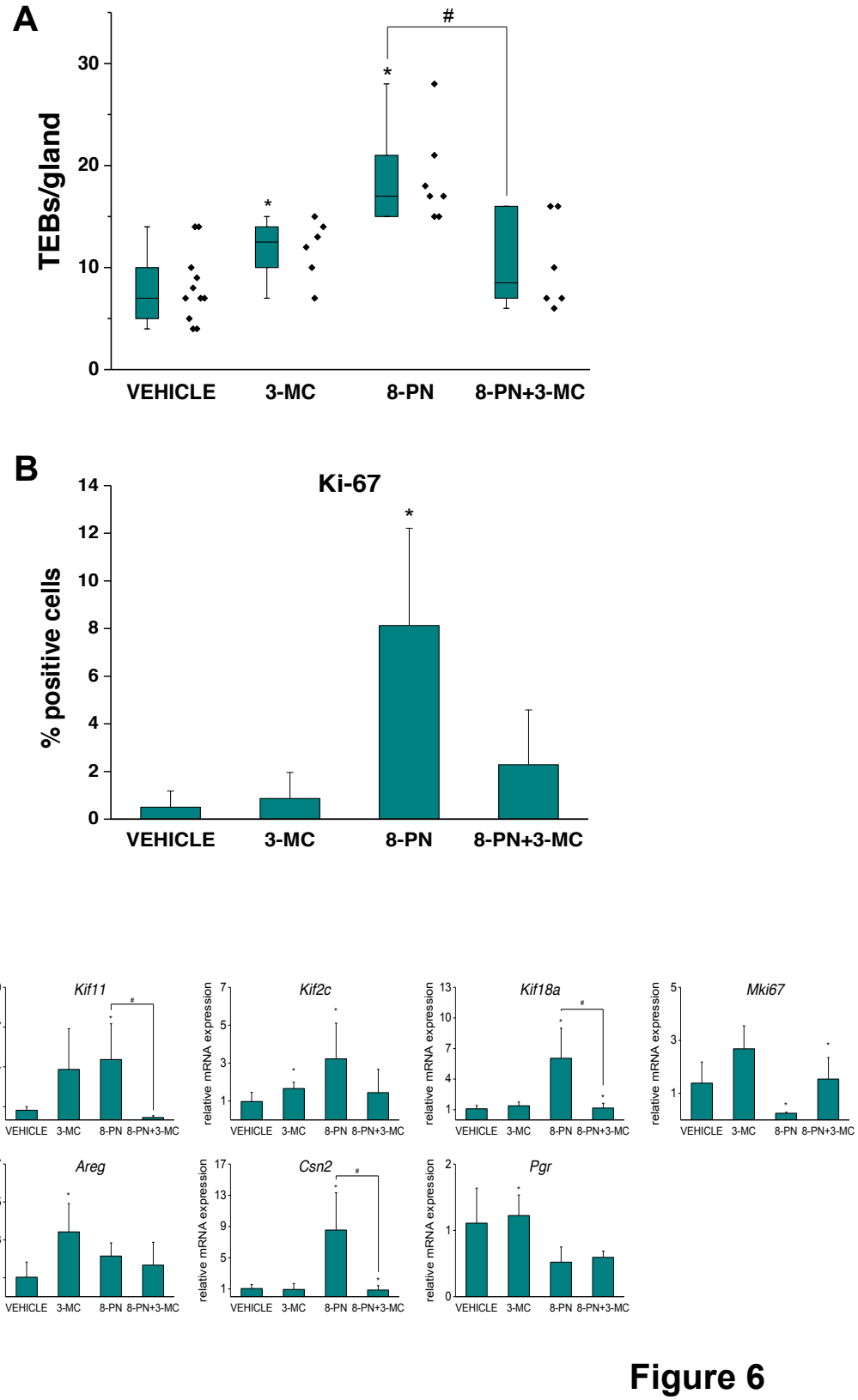
A
TEBs/gland
VEHICLE
3-MC
8-PN
8-PN+3-MC
B
Ki-67
% positive cells
C
Kif11
Kif2c
Kif18a
Mki67
Areg
Csn2
Pgr
relative mRNA expression

Figure 6